Descriptive Report

Pharmaceutical product comprising tissue of the male vegetal reproductive system

Field of the Invention

The present invention is related to pharmaceutical products and the production processes therefor. More specifically, this invention is related to pharmaceutical products comprising pollen grains having altered protein composition through genetic modification in the plant that produces them. The production processes of the pharmaceutical products of the present invention comprise the cultivation of genetically modified plants having pollen grains with altered molecular composition, making them useful for the treatment of allergies, autoimmune diseases, as well as the vaccination of eucarytic organisms, and for *in vitro* diagnosis applications.

Background of the Invention

The processes to obtain economically relevant compounds have been changing significantly in recent years in the area of biotechnology. Until recently, the traditional industrial fermentations, for instance, were the main technological option for the production of polypeptides, enzymes, antibiotics and other substances of economical interest. Meanwhile, as the knowledge about microbiology, biochemistry and genetics of the organisms involved in the fermentative processes increased, the production technologies have also been adapted and modified. The development of molecular biology techniques was remarkable in this context: besides offering tools for the understanding of the biochemical, genetic and evolutionary mechanisms of the many species studied, molecular biology also furthered the development of countless alternatives for the industrial production of substances, as a result of the combination possibilities of the different organism's characteristics.

The present-day market shows a great demand of products and processes in the Human and/or Animal Health segment and the interest in the

development of technologies in this area continues. From the industrial technology standpoint, many factors can be considered as limiting or technological "bottlenecks". Especially relevant in the production of economically important substances are the costs involved in the purification, which frequently demands a series of complex and costly steps. The determination of the cost of new therapeutic products is a very complex process, if all phases involved are to be considered – from the product conception until its placement on the market. One major factor of impact on the costs is the adopted technology, especially in the case of biological products whose chemical synthesis has not yet been developed or which presents technical or economical disadvantages. The advance of the recombinant DNA technology allowed the production of proteins and other substances in virtually unlimited quantities. On the other hand, the expression system employed has a remarkable influence on the product's nature and on the production process. A good illustrative example is the production of the Tissue Plasminogen Activator (tPA), an anti-thrombotic agent used in the treatment of myocardium infarct, thrombosis and pulmonary embolism. This substance, when produced by *Escherichia coli*, or through the cultivation of genetically modified ovary cells from hamsters (CHO), presents significant differences regarding its structure (glycosilation, need of renaturing etc.) and production processes, which reflects on its price. In *E. coli*, 12% of the tPA's production costs correspond to the fermentative phase of the process and 88% are due to purification. On the other hand, in the production through CHO, 75% of the costs derive from the cellular cultivation and 25% from purification (Datar *et al*, "Process Economics of Animal Cells and Bacterial Fermentations: A Case Study Analysis of Tissue Plasminogen Activator" *Bio/Technology* **11**:349-357, 1993). These differences are significant and can be decisively influent in the choice of the process to be used. Still other factors have a special importance in the choice of the production system, such as funds (capital) for the factory's installation, costs of raw materials, the need of qualified personnel (and its cost) as well as environmental and safety aspects.

The Vegetal Model

A safer and cheaper system to produce biologically originated substances may be idealized in transgenic plants cultivated in contained areas (greenhouses) or in agricultural areas. Furthermore, the vegetal system to produce substances of pharmaceutical interest offers various advantages, among which one might mention the absence of animal viruses and other animal cell products, as well as the absence of the typical contamination of bacterial fermentative processes, from yeasts or animal cells. In this context, the knowledge of the genetic and biochemical mechanisms of some vegetal models is reasonably wide, standing out, among other plants, *Arabidopsis thaliana, Nicotiana tabacum* and *Oryza Sativa*. Among the main advantages of the use of the vegetal system for the production of substances with economical interest, one should emphasize the easier scale up, which is fundamental in the case of polypeptides and/or proteins of industrial interest. In the case of using microorganisms (recombinant or not), the phases involved in the scale up are normally the limiting factors of the process economy. This occurs for various reasons, among which the non-linearity of the determining relations of the process' efficiency (oxygen transfer, rheological factors, energy demand of the process etc.), the cost of the necessary production equipments and the need of adequately qualified personnel. In the case of the vegetal system, the mentioned cost components are much lower, since the scale-up tends to be simpler and linear. Besides that, in the evaluation of an industrial process' economy, the strategies seeking to eliminate the largest possible number of purification phases should be considered. Furthermore, plants are metabolically able to perform complex post-translational modifications, such as glycosilations, which widens the scope of possible peptides, antigens or vaccine candidates to be produced by plants. In this sense, the strategy of the present invention comprises the development of expression systems which take into account the most appropriate cellular and sub-cellular localizations for the desired products and, foremost, the elimination of purification steps.

The reproductive systems of *A. thaliana* and *N. tabacum* have been intensely studied in the recent years and are suitable to the logic of eliminating purification steps of the present invention. The starting point for the idealization of the present invention was the existing knowledge about the genes involved in the vegetal reproductive development and, more precisely, those related to the development of the tapetum which, for some years now, is the study object of the Laboratory for Plant Molecular Genetics of the Federal University of Rio de Janeiro, Brazil. Former studies in that laboratory in this area comprise the state of the art of the present invention. On one hand, the knowledge about the expression of the genes involved in the formation of *A. thaliana's* inflorescence (Franco, MSc. Dissertation, UFRJ, 1992) was helpful to understand the function of the oleosin-type proteins, more recently studied with molecular biology techniques associated to microscopy (Ferreira, PhD Thesis, UFRJ, 1997). On the other hand, there also exists knowledge about the regulation mechanisms of the codifying genes of sadi oleosin-type proteins, studied with the help of the β-glucuronidase (GUS) gene marker (Scholte, PhD Thesis, UFRJ, 1998). Even more recently, a strategy of modifying the protein composition of the external surface of pollen grains was described by Foster *et al* "Modifying the pollen coat protein composition in Brassica", *Plant Journal* **31**(4): 477-486, 2002, also described in the document WO 99/49063, by the same authors. However, neither these, nor any other reference known by the author make any allusion or suggestion regarding the use of genetically modified plants for the production of pharmaceutical products in tissues and/or cells of the male vegetal reproductive system, neither do they mention the use of whole, intact, pollen grains derived from genetically modified plants as pharmaceutical products to be used in immunoreactions, as vaccines or as reactive agents for diagnostics, which collectively comprise the objects of the present invention.

For the purposes of the present invention, one should understand as "tissues and cells of the male vegetal reproductive system" the tissues or cells of the male vegetal reproductive system, including the anthers, tapetum, pollen grains, parts and combinations thereof. For the the purposes of the present

invention, one should also understand as "immunoreactions" all reactions that involve cells and/or molecules of the immune system of eukaryotes, including veterbrates, invertebrated, mammals and the like, including mononuclear cells such as macrophages and lymphocytes B and T, neutrophils, eosinophils, besides antigens, antibodies, cytokines and other chemical mediators of the immune system, including parts of the same and its combinations. Furthermore, for the purposes of the present invention, one should understand as "heterologous polypeptide" any aminoacid sequence which is not naturally produced by the plant, but whose synthesis in it derived from the genetic modification undertaken in the plant through the present invention. Without limiting the scope of the present invention, one should emphasize as being of special importance the use of pollen grains containing at least one heterologous polypeptide.

The present invention offers means to avoid some difficulties in both the production and use of polypeptides with therapeutic and/or diagnostics interest. On one hand, pollen grains are structurally stable, probably as a consequence of the need of reproductive success. Therefore, their "evolution" in order to resist the most diverse environmental stresses is useful to the logic of using them as a product, since their high stability is favorable and desirable. In an additional aspect, whole pollen grains of the present invention may be used in certain applications, bypassing the need of purification. As examples, herein used to illustrate the present invention but not to limit its scope, whole pollen grains containing heterologous polypeptides would permit: the direct use as antigens in the production of diagnostic kits, especially for screening, and its use as a vaccine preferably delivered onto mucous membranes or injected subcutaneously.

Pollen is often associated to allergy because some people develop symptoms such as sneezing, itching, cough, nasal irritation, eye watering and asthma when exposed to the pollen of certain plants. These particles are carried in large amounts by the air, normally during springtime. When they get in contact with the nasal mucous membranes or the throat, they may trigger

allergic reactions known as polinosis or seasonal allergic rhinitis (for more details, consult Balda *et al*, "Tree-pollen Allergy is Efficiently Treated by Short-term Immunotherapy (STI) with Seven Preseasonal Injections of Molecular Standardized Allergens". *Allergy* **53**, 740-748, 1998). As in any allergic process, the polinosis is a high sensibility to certain substances present in the pollen, and considerably varies from person to person, even though there seems to be a familiar correlation. In the majority of allergic reactions, the immune system responds to a "false alarm", mobilizing the attack against the allergen. The organism produces large quantities of specific IgEs, which bind themselves to the mastocytes in the tissues and to the basophils in the blood. When the allergen meets the IgE the liberation of histamine, prostaglandin, leucothriens and other substances occurs, thus causing the allergy symptoms (for more details, see Batanero *et al*, "IgE-binding and Histamine-release Capabilities of the Main Carbohydrate Component Isolated from the Major Allergen of Olive Tree Pollen, Ole e 1". *J Allergy Clin Immunol* **103**, 147-153, 1999). Some strategies have been developed in order to obtain vaccines against autoimmune diseases and allergies, being generally based on the induction of tolerance. For example, known pollen grain allergens, when prepared in an encapsulated form, have shown to be efficient in the induction of tolerance by means of nasal administration. Nevertheless, the available encapsulating methods of allergens/antigens are laborious and costly, besides generally bringing about the denaturation of said allergens/antigens. The present invention's approach makes it possible to overcome these problems through the production of heterologous allergens/antigens in pollen grains.

Due to its natural ability to stimulate the production of specific IgEs and IgGs, we started from the hypothesis that the pollen could become a good candidate as a carrier and deliverer of vaccine antigens in mucous membranes, such as its direct use via nasal immunizations. Besides the already mentioned elevated structural stability of the pollen, an additional characteristic of the present invention under consideration is to enable the use of whole pollen grains as vaccines. Interestingly, one of the goals clearly expressed by the

World Health Organization (WHO) is the development of new systems for the delivery of antigen vaccines to the respiratory tract. Furthermore, recent studies have demonstrated that vaccines derived from transgenic plants, when applied in the form of dry powder, seem to be the preferred solution for the lack of homogeneity in the concentrations of antigens produced in plants (for further references, see Sala *et al.*, "Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives". *Vaccine* **21**:803-808, 2003; Mielcarek *et al.* "Nasal vaccination using live bacterial vectors". *Advanced Drug Delivery Reviews* **51**:55-69, 2001). On the other hand, the WHO's recommendation for the vaccine against tetanus (or *lockjaw*), for instance, is the administration of three consecutive doses of the respective antigen. This repeated administration has financial and logistical disadvantages, since some patients do not return for the second dose and because the vaccination campaigns have as a limiting factor: the need of a cold chain, considering that practically all vaccines are thermo labile. The availability of vaccines in pollen grains presents itself as capable of solving these mentioned problems, besides being easily delivered (without injections), easily standardized and endowed with an elevated thermal stability, being able to help avoid the logistics problems which are so far inherent to vaccination campaigns. These advantages open up good perspectives for the study and development of this way of immunization in combination with the presentation form of the present invention.

Several efforts have been made in the last two decades in the sense of trying to develop subunit vaccines for human and veterinary use. The subunit vaccines are based on individual components derived from the infectious agent and, normally, have a low immunogenicity due to the absence of other cellular constituents from which they are often purified. Therefore, when developing vaccines it is desirable to plan the utilization of other substances which have the potential to increase the immune response to the antigens in question, what is normally done with the use of adjuvants. Entire cells or parts thereof can work as self-adjuvants, which is favorable for the present invention's approach. The

identification of an appropriate antigen is only the first step in the development process to obtain a subunit vaccine, since adequate adjuvant systems and delivery systems of the respective antigen are also necessary. An adjuvant can be any material which increases the immune-humoral and/or cellular response to the antigen(s); it is generally accepted in literature that certain adjuvants act through the gradual liberation of the antigens to the cells of the immune system. Recent studies (Wiedermann, *et al.* "Modulation of an allergic immune response via the mucosal route in a murine model of inhalative type-I allergy" *Intl. Arch. Allergy Immunol.* **118**:129-132, 1999) have shown that antigenic preparations in powdered form can also increase the incorporation of antigens by the antigen processing cells of the immune system. In this sense, the use of whole pollen grains, due to its powdered nature, offers this additional advantage in the case of application in vaccines. Independently of exactly knowing the specific mechanism involved, it is known that not only the cellular, but also the humoral immunity might be stimulated in various degrees, depending on the antigen, the adjuvant, the administration protocol and the species involved.

In order to develop an effective and commercially feasible vaccine, the relation between the production cost and the large-scale production capacity of the antigenic preparation and the adjuvant system should be taken into account. On the other hand, the growing number of vaccines under developed and the number of required injections for a wide-ranging immunization program for children, for instance, generates elevated costs and the preoccupation about the discontinuity potential of current vaccination programs. This makes highly desirable the availability of alternative immunization means, especially those allowing the so-called multivalent vaccination, that is, the one that permits to present multiple antigens or epitopes simultaneously. In this context, transgenic plants have been the object of several attempts to obtain "edible" vaccines, due to their notorious advantages in terms of production costs and administration. However, vaccines produced in transgenic plants destined for oral consumption have the disadvantage of passing by the gastrointestinal barrier, which destroys a significant portion of the vaccine antigens. For that reason, the state of the art

shows that the amount of antigens required for an effective immune response derived from an edible transgenic plant vaccine is from 100 to 1,000 times higher than the amount of antigens necessary for an effective parenteral immunization (for further reference, see Carter III, J.E., and Langridge, W.H.R. "Plant-based vaccines for protection against infectious and autoimmune diseases". *Critical Reviews in Plant Sciences* **21**(2), 93-109, 2002; Streatfield and Howard "Plant-based vaccines". *International Journal for Parasitology* **33**, 479-493, 2003). Therefore, the need to develop new alternatives to overcome this difficulties persists.

The entry route of most pathogenic agents is the mucous surfaces and a large part of the infections is located in the mucous and sub mucous tissues. However, conventional injectable vaccines are poorly efficient for the induction of an immune response in mucous membranes. As a consequence, several experiments are being carried out in order to offer alternatives for the immunization in mucous membranes, which includes, for instance, the incorporation of antigens in larger particles (such as liposomes, immune stimulatory complexes, microspheres) for increasing the efficiency of the immune response in mucous tissues. On the other hand, the use of adequate adjuvants is normally very important for the stimulation of the desired type of immune response. Among the known adjuvants, and in the scope of the present invention, one might point out the vegetable oils. A primary advantage of the use of vegetable oil adjuvants over the use of mineral oils is that vegetable oils can be easier metabolized and are, therefore, more tolerable. Patent literature is rich in examples of the use of vegetable oils as adjuvants in vaccines, including, for instance, documents WO 01/95934 "The use of plant oil-bodies in vaccine delivery systems" and WO 02/00169 "Production of vaccines using transgenic plants or modified plant viruses as expression vectors and transencapsidated viral coat proteins as epitope presentation systems". Most of these patents disclose formulations and the use of water-in-oil or oil-in-water emulsions which are prepared through the mixture of pure chemical compounds. However, none of the mentioned documents reveals or suggests

the use of tissues or cells from the male vegetal reproductive system, such as whole pollen grains, or even parts thereof, as an useful pharmaceutical product in immune reactions or as an antigen-adjuvant combination for immunization, preferably applicable in mucous membranes. In this context, the present invention offers a technical and commercially feasible production alternative of a pharmaceutical product which is potentially useful in the treatment of dysfunctions of the immune system of eucaryotes and which allows, among other characteristics, the production of multiple substances in one single system.

Summary of the Invention

Having in mind the prior art limitations and all the above-mentioned reports, the present invention provides pharmaceutical products as well as their production process in genetically modified plants. The products of the present invention comprise tissues and/or cells of the male vegetal reproductive system, including bioactive substances to be used in Human and/or Animal Health.

In one aspect, the present invention provides a production process of pharmaceutical products which overcome difficulties in the production, distribution, storage and logistics of said products. In another aspect, the present invention offers a production process of pharmaceutical products to be used as immune reactions, through the expression, in male vegetal reproductive tissues, of marking genes, codifying genes of antigens and/or therapeutic polypeptides. In yet another aspect, the pharmaceutical products of the present invention are useful in the immune modulation of eukaryotes, including mammals and other vertebrates as well as invertebrates such as insects and the like. From an additional point of view, the pharmaceutical products of the present invention are potentially useful in immune diagnostic reactions. These and other aspects constitute the objects of the present invention and will be clearer through their detailed description and the corresponding attached figures, as well as the appendend claims.

Detailed Description of the Invention

In order to evaluate the applicability of the use of tissues and cells from the male vegetal reproductive system as pharmaceutical products, we evaluated situations which could be considered to be most critical to said use. Therefore, the tested applications, even though not limiting the scope of the present invention, are herein presented as the use of whole pollen grains as vaccines, immunotherapic agents and/or diagnostics reagents. In order to achieve this goal, genetic constructions were developed aiming to allow the expression of a reporter gene in such a way that its presence in plant subcellular structures and in pollen grains could be more easily analyzed. Different genetic constructions containing the reporter gene *GUS* (codifier of the β–glucuronidase enzyme) were evaluated: (i) the binary vector pDE1001 for the transformation of *N. tabacum* plants containing the reporter gene *GUS* under the control of the promoter PvChit (promoter of the bean chitinase, *Phaseus vulgaris*, the genetic construction of which was detailed in Lima *et al.*, (2002) Bean class IV chitinase promoter is modulated during plant development and under abiotic stress. *Physiologia Plantarum* **116**:512-521, in a construction herein called pPvchitΔ1200*gus,* Figure 1), which allows the intracellular expression of the glucuronidase (GUS) enzyme in tobacco pollen grains; and (ii) the binary vector for the transformation of plants pCambia 1303 (Figures 4 and 6) as the receptor of the fusions At*GRP17-GUSGFP* under the control of the promoter of a glycine-rich protein – AtGRP17 – from *A. thaliana* (formerly called AtGRP7 by de Oliveira *et al.* "Inflorescence-specific genes from *Arabidopsis thaliana* encoding glycine-rich proteins". *Plant J.* **3**:495-507, 1993). This last construction aims to permit the expression of the fusion AtGRP17-GUSGFP on the surface of pollen grains, in order to allow the testing of the pollen grains hereby obtained for the applications of the present invention.

Example 1 – Obtaining "WT" and "GM" tobacco pollen grains

Wild tobacco plants (*N. tabacum*) from the SR1 lineage, referred to as "WT" in the present invention, and also tobacco plants transformed with the

vector pPvchitΔ1200*gus* (Figure 1), available from a previous study (Viviane Moreira, PhD Thesis, UFRJ, 2002, also described in Lima *et al.*, "Bean class IV chitinase promoter is modulated during plant development and under abiotic stress". *Physiologia Plantarum* **116**:512-521, 2002) were cultivated in a greenhouse until the blossoming of mature flowers. The genetically modified lineage of tobacco in the present invention is referred to as "GM", and produces pollen grains with an altered composition, containing the *GUS* enzyme in its interior. The following procedure was adopted in order to collect the pollen grains: as soon as the dehiscence occurred, the entire flowers (Figure 2) were removed from the WT and GM plants and the intact anthers were transferred into Eppendorf tubes with the help of a scalpel. The average weight of the pollen grains present in each inflorescence was weighted with an analytical scale. Taking into account the difference between the total weight with anthers and the total weight without anthers (after homogeneization and the subsequent removal of the pollen grains present in said anthers), the average weight of pollen grains present in each flower was equal to 1 milligram – consequently, the equivalent of 0.2 milligram for each anther, since one *N. tabacum* flower has 5 (five) anthers.

Example 2 – Evaluation of GUS stability in "GM" pollen grains

The stability of pollen grains maintained at room temperature (about 25 degrees Celsius) was evaluated qualitatively through the verification of the activity of the β glucuronidase (GUS) enzyme in the GM pollen grains. In order to do so, colorimetrical reactions were performed using 1 μL of the compound X Gluc in 100 μL of phosphate buffer 0.1 M pH 7 and whole transgenic pollen grains containing the GUS enzyme. In the tests performed in laboratory, the activity of the GUS enzyme was kept in stock for at least one year at room temperature. In this context, it is worthwhile to emphasize that the physico-chemical conditions necessary for the maintenance of an enzyme's activity are much more restricted than the conditions necessary to maintain the immunogenicity of a polypeptide sequence. Therefore, these results suggest an

elevated stability of heterologous peptides expressed in the pollen grains of *N. tabacum* "GM", which is desirable within the scope of applications of the present invention.

Analysis of the immune response of rats to tobacco pollen grains

Since the present invention relates to new production technologies and formulations of pharmaceutical products especially applicable in immune reactions, such as immune modulators, vaccines and diagnosis reagents, the immunoreactivity of pollen grains under different conditions is the starting point for the evaluation of its applicability. The approach used herein includes the study of the immune response of female Wistar rats (*Ratus novergicus*), submitted to different ways of pollen grain exposures.

The potential of pollen grains to induce inflammation was evaluated by analyzing different administration schedules of WT tobacco pollen grains in rats. The cellular responses of animals submitted to different exposure/sensitizing schemes were monitored by counting the amount of cells present in broncho-alveolar lavage or in pleural fluid and checking the appearance of paw edemas, while the molecular responses were monitored through the observation of protein extravasation, measurement of the total serological IgE and IgG concentrations through ELISA, and of specific serological IgGs against pollen grain proteins by Western Blot.

Groups of 3 to 6 animals, as indicated, were submitted to the administration of different regimes of saline solution suspensions containing WT or GM tobacco pollen grains, in the concentrations of 0.5, 50 and 500 µg per animal (concentrations based on the average weight of 1 mg of pollen per tobacco flower), with or without the joint administration of alumminum hydroxide (5 mg per animal) as adjuvant. The verification of the occurrence or not of any inflammation in the broncho-alveolar cavity or the pleural cavity was done respectively through broncho-alveolar or pleural washing, followed by the total leucocytes count in a Neubauer chamber and a relative count of monocytes, neutrophils and eosinophils after centrifugation on a glass slide. Both counts

were carried out with the help of an optical microscope. As will become shown in the following experiments, none of the tested conditions caused a significant alteration of cell counts consistent with an inflammatory process. Additionally, none of the tested conditions produced significant alteration of the protein exudates, which would be typical of an inflammatory process. Serum samples of the animals submitted to the different experimental conditions were collected and stored for later molecular analysis. And again, none of the pollen grain administration conditions tested showed a significant increase of the total serological IgE and IgG concentrations, according to quantitative measurements through ELISA. The results of the following tables show that none of the different pollen grain administration conditions caused detectable allergic/inflammatory type reactions in animals.

Example 3 – Immune response of rats submitted to a single intrapleural injection

Table 1 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. The animals were submitted to an intrapleural injection of 5 μg of wild tobacco pollen grains (100 μL in saline solution). The chart shows the cell counts in pleural lavage 4 hours after the procedure.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	17	85	15	0	4.34	0.77	0
2	25	83	17	0	6.23	1.28	0
3	16	85	15	0	4.08	0.72	0
Average					4.88	0.92	0
Deviation					1.17	0.31	0
E.P.M					0.83	0.22	0

Subtitle abbreviations: CAM Leu tot (total leucocytes counted in a Neubauer chamber); Mono % (% of monocytes); Eos % (% of eosinophils); Neut % (% of neutrophils); E.P.M. (mean standard error).

Table 2 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. The animals were submitted to an intrapleural injection of 50 μg of wild tobacco pollen grains (100 μL in saline solution). The chart shows the cell counts in pleural lavage 4 hours after the procedure.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	17	86	14	0	4.39	0.71	0
2	12	78	22	0	2.81	0.79	0
3	17	88	11	1	4.49	0.56	0.05
Average					3.89	0.69	0.02
Deviation					0.94	0.12	0.03
E.P.M					0.67	0.08	0.02

Table 3 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. The animals were submitted to an intrapleural injection of 500 μg of wild tobacco pollen grains (100 μL in saline solution). The chart shows the cell counts in pleural lavage 4 hours after the procedure.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	20	82	18	0	4.92	1.08	0
2	21	81	19	0	5.10	1.20	0
3	23	89	11	0	6.14	0.76	0
Average					5.39	1.01	0
Deviation					0.66	0.23	0
E.P.M					0.47	0.16	0

The results of tables 1 - 3 show that in none of the tested conditions a statistically significant alteration of the cell counts in pleural lavage occurred,,indicating that the administration of pollen grains under these conditions does not induce the characteristic inflammatory response of allergies. In view of these results, a new battery of experiments was conducted by using a larger number of animals and a higher exposure to the pollen grains.

Example 4 – Immune response of rats submitted to consecutive intrapleural injections

Table 4 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Saline solution intrapleural injection (as control, 100 μL per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in pleural

lavage 4 hours after the procedure by intrapleural injection of 5 μg of pollen grains in a volume of 100 μL per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	44	78	3	19	11.67	0.45	2.84
2	30	78	17	5	7.02	1.53	0.45
3	24	77	16	7	5.54	1.15	0.50
Average					8.08	1.04	1.27
Deviation					3.20	0.55	1.37
E.P.M					2.26	0.39	0.97

Table 5 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intrapleural injection of 5 μg of wild tobacco pollen grains (in saline solution injection of 100 μL per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in pleural lavage 4 hours after the procedure by intrapleural injection of 5 μg of pollen grains in a volume of 100 μL per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	27	85	13	2	6.89	1.05	0.16
2	45	77	8	15	10.40	1.08	2.03
3	52	64	11	25	11.32	1.94	4.42
4	85	69	8	23	19.94	2.31	6.65
Average					12.13	1.60	3.31
Deviation					5.54	0.63	2.82
E.P.M					3.20	0.36	1.63

Table 6 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intrapleural injection of 50 μg of wild tobacco pollen grains (in saline solution injection of 100 μL per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in pleural lavage 4 hours after the procedure by intrapleural injection of 50 μg of pollen grains in a volume of 100 μL per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	50	68	14	18	10.20	2.10	2.70
2	37	69	14	17	7.66	1.55	1.89
3	145	71	16	13	37.06	8.35	6.79
4	36	81	10	9	8.75	1.08	0.97
Average					15.92	3.27	3.09
Deviation					14.13	3.41	2.57
E.P.M					8.16	1.97	1.48

Table 7 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intrapleural injection of 500 μg of wild tobacco pollen grains (in saline solution injection of 100 μL per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in pleural lavage 4 hours after the procedure by intrapleural injection of 500 μg of pollen grains in a volume of 100 μL per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	67	77	7	16	19.60	1.78	4.07
2	86	55	7	38	16.08	2.05	11.11
3	136	56	17	27	30.46	9.25	14.69
4	147	43	4	53	25.28	2.35	31.16
Average					22.86	3.86	15.26
Deviation					6.33	3.60	11.48
E.P.M.					3.66	2.08	6.63

The results of tables 4 - 7 show that there was no statistically significant alteration of the cell counts in pleural lavage under the conditions of consecutive administrations of 5 and 50 μg of pollen grains. In the condition of administrating 500 μg of pollen grains there was a significant increase in the counts of mononuclear cells and neutrophils, as well as an increase (even though statistically not significant) of eosinophils. These results are also shown in figure 3, panels A, B and C, respectively. The observed increase of the cell counts in the animals submitted to the administration of 500 μg of pollen grains demonstrates that the animals are responsive only to extreme concentrations of said pollen grains. However, the intrapleural administration of pollen grains in the concentration range of up to 50 μg per animal, in 3 consecutive applications did not induce detectable inflammatory-type response which is typical of allergies. Since the typical conditions of intrapleural administration of antigens very rarely exceed a concentration of 20 μg per animal, these results show that intrapleural pollen grain injections could be, surprisingly, employed in immunization or immunotherapy programs for animals, without hereby inducing the appearance of typical allergic/inflammatory symptoms. In order to verify the

response of animals submitted to intranasal administration of pollen grains, another set of experiments was performed, as shown in the tables below.

Example 5 – Immune response of rats submitted to two consecutive intranasal instillations

Table 8 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intranasal instillation of saline solution (as a control, 50 μL per nostril per animal) in two consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage of normal animals, 4 hours after the last instillation.

N	CAM Leu tot	Mono cont.	Eos cont.	Neut cont.	Mono thousands	Eos Thous.	Neut Thous.
1	74	73	9	18	1351	167	333
2	28	89	0.5	10.5	623	4	74
3	45	99	1	0	1114	11	0
4	26	30	0	70	195	0	455
Average					820.6	45.3	215.4
Deviation					515.5	80.9	214.3
E.P.M.					297.6	46.7	123.7

Table 9 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intranasal instillation of 5 μg of wild tobacco pollen grains (in saline solution, instillation of 50 μL per nostril per animal) in two consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	127,5	54	23	23	1721	701	765
2	64	70	0	30	1120	0	480
3	81	71	0	29	1438	0	587
4	41	97	0	3	994	0	31
5	38,5	85	0	15	818	0	144
Average					1218.3	140.3	401.5
Deviation					360.9	313.6	306.7
E.P.M.					180.5	156.8	153.4

Table 10 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intranasal instillation of 50 μg of wild tobacco pollen grains (in saline solution, instillation of 50 μL per nostril per

animal) in two consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	223	46	0	54	2565	0	3011
2	147	62	1	38	2279	37	1397
3	37	95	0	5	879	0	46
4	30	97	0	3	728	0	23
5	68	100	0	0	1700	0	0
Average					1629.9	7.4	895.2
Deviation					943.7	18.4	1323.8
E.P.M.					409.1	8.2	661.9

Table 11 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intranasal instillation of 500 μg of wild tobacco pollen grains (in saline solution, instillation of 50 μL per nostril per animal) in two consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	68.5	70	0	30	1199	0	514
2	48	77	0	23	924	0	276
3	86	62	0	38	1333	0	817
4	135	62.5	0	37.5	2109	0	1266
Average					1391.3	0	718.1
Deviation					508.1	0	426.9
E.P.M.					293.3	0	246.5

Example 6 – Immune response of rats submitted to three consecutive intranasal instillations

Table 12 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intranasal instillation of saline solution (as a control, 50 μg per nostril, per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage of normal animals.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	49	83	0	17	1017	0	208
2	35	96	0	4	840	0	35
3	69	59	0	41	1018	0	707
4	47	84	0	6	987	0	71
5	58	57	0	43	827	0	624
Average					937.6	0	328.9
Deviation					96.2	0	315.3
E.P.M.					48.1	0	157.6

Table 13 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intranasal instillation of 5 μg of wild tobacco pollen grains (in saline solution, instillation of 50 μL per nostril, per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.

N	CAM Leu tot	Mono cont.	Eos cont.	Neut cont.	Mono Thous.	Eos Thous.	Neut Thous.
1	79	53	0	47	1047	0	928
2	60	60	4	36	900	60	540
3	53.6	31	0	69	415	0	925
4	103	62	0	38	1597	0	979
Average					989.7	15	842.8
Deviation					486.3	30	203.4
E.P.M.					280.7	17.3	117.4

Table 14 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intranasal instillation of 50 μg of wild tobacco pollen grains (in saline solution, instillation of 50 μL per nostril, per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.

N	CAM Leu tot	Mono %	Eos %	Neut %.	Mono Thous.	Eos Thous.	Neut Thous.
1	119	37	0	63	1101	0	1874
2	93	88	0	12	2046	0	279
3	66	97	0	3	1601	0	50
4	86	77	3	20	1656	65	430
Average					1600.7	16.1	658.2
Deviation					387.8	32.3	825.7
E.P.M.					223.9	18.6	476.7

Table 15 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Intranasal instillation of 500 μg of wild tobacco pollen grains (in saline solution, instillation of 50 μL per nostril, per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	83	50	0	50	1038	0	1038
2	90	82	0	18	1845	0	405
3	104	98	0	2	2548	0	52
4	53	47	0	53	623	0	702
5	45	66	0	34	743	0	383
Average					1359.2	0	515.9
Deviation					817.8	0	371.5
E.P.M.					408.9	0	185.8

The results of tables 12 – 15 show that in none of the tested conditions a statistically significant alteration of the cell counts in broncho-alveolar lavage occurred, indicating that the administration of pollen grains under these conditions does not induce detectable allergic-type inflammatory response. In view of these results, a new set of experiments was conducted involving a larger number of animals and higher concentrations of pollen grains. These results are also presented in figure 3, panels A, B and C, respectively, which clearly show that there was no statistically significant alteration of the cell counts in pleural lavage under the consecutive administration conditions of 5, 50 and 500 μg of pollen grains.

Example 7 – Immune response of rats subjected to subcutaneous injection, followed by a "booster" – challenge with 10 μg of pollen grains in the pleural cavity

Table 16 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. The chart shows the cell counts in pleural lavage of normal animals, which were not submitted to any program of sensitizing or challenge.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	39	87	12	1	11.5	1.6	0.1
2	34	81	19	0	8.3	1.9	0
3	26	89	11	0	7.4	0.9	0
4	33	77	23	0	7.6	2.3	0
5	40	86	13	1	11.7	1.8	0.1
Average					9.3	1.70	0.05
Deviation					2.13	0.51	0.07
E.P.M.					1.07	0.25	0.04

Table 17 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Saline solution subcutaneous injection (according control, 100 μL per animal), followed by a *booster* in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the *booster*, that is, on the 14th day) through intrapleural injection of 10 μg of pollen grains in a volume of 100 μL of saline solution per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	29	67	33	0	6.6	3.3	0
2	53	24	5	71	3.9	0.8	11.7
3	39	63	36	1	7.4	4.2	0.1
Average					5.97	2.76	3.93
Deviation					1.8	1.75	6.70
E.P.M.					1.27	1.24	4.74

Table 18 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Subcutaneous injection of 50 μg of wild tobacco – WT – pollen grains (100 μL per animal, in saline solution), followed by a *booster* in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the *booster*, that is,

on the 14th day) through intrapleural injection of 10 μg of pollen grains in a volume of 100 μL of saline solution per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	31	51	35.5	13.5	6	4.2	1.6
2	24	80	17.5	2.5	6.1	1.3	0.2
3	29	63	30.5	6.5	5.5	2.7	0.6
4	26	50	46	4	3.9	3.3	0.6
5	28	82	14	4	7.3	1.3	0.4
Average					5.78	2.54	0.67
Deviation					1.25	1.26	0.54
E.P.M.					0.63	0.63	0.27

Table 19 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Subcutaneous injection of 500 μg of wild tobacco – WT – pollen grains (100 μL per animal, in saline solution), followed by a *booster* in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the *booster*, that is, on the 14th day) through intrapleural injection of 10 μg of pollen grains in a volume of 100 μL of saline solution per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	48	49	6	45	8	0.98	7.34
2	47	53	10	37	8.97	1.69	6.26
3	61	76	5	19	14.8	0.98	3.71
4	33	89	10	1	8.8	0.99	0.10
5	41	62	3	35	8.6	0.42	4.88
Average					9.85	1.01	4.46
Deviation					2.81	0.45	2.80
E.P.M.					1.41	0.23	1.40

Table 20 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Subcutaneous injection of 50 μg of wild tobacco – WT – pollen grains (100 μL per animal, in saline solution with 5 mg of alumminum hydroxide as adjuvant), followed by a *booster* in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the *booster*, that is, on the 14th day) through intrapleural injection of 10 μg of pollen grains in a volume of 100 μL of saline solution per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	49	77	8	15	13.58	1.41	2.65
2	23	76	17	7	5.94	1.33	0.55
3	49	73	13	14	11.09	1.97	2.13
4	33	82	15	3	8.12	1.49	0.30
5	56	56	15	29	9.41	2.52	4.87
Average					9.63	1.74	2.10
Deviation					2.90	0.50	1.85
E.P.M.					1.45	0.25	0.92

Table 21 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Subcutaneous injection of 500 μg of wild tobacco – WT – pollen grains (100 μL per animal, in saline solution with 5 mg of aluminum hydroxide as adjuvant), followed by a *booster* in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the *booster*, that is, on the 14th day) through intrapleural injection of 10 μg of pollen grains in a volume of 100 μL of saline solution per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	47	67	2	31	11.34	0.34	5.25
2	94	62	5	33	22.15	1.79	11.79
3	32	68	3	29	6.96	0.31	3
4	56	69	4	27	12.36	0.72	4.84
5	52	71	18	11	11.08	2.81	1.72
Average					12.78	1.19	5.31
Deviation					5.63	1.08	3.89
E.P.M.					2.81	0.54	1.95

Example 8 – Immune response of rats subjected to subcutaneous injection, followed by a "booster" – challenge with 20 μg of pollen grains in the pleural cavity

Table 22 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Subcutaneous injection of saline solution (according to control 100 μL per animal), followed by a *booster* in the

same conditions after 7 days. The chart shows the cell counts in pleural lavage of normal animals, which were not subjected to any program of sensitizing or challenge.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	15	89	11	0	4.3	0.5	0
2	25	84	16	0	6.3	1.2	0
3	14	71	25	4	3.2	1.1	0.2
4	21	88	12	0	5.9	0.8	0
Average					4.92	0.91	0.04
Deviation					1.45	0.31	0.09
E.P.M.					0.84	0.18	0.05

Table 23 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Subcutaneous injection of saline solution (according to control 100 μL per animal), followed by a *booster* in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the *booster*, that is, on the 14th day) through intrapleural injection of 20 μg of pollen in a volume of 100 μL of saline solution per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	34	69	31	0	7.0	3.2	0
2	21	81	18	1	5.3	1.3	0.1
3	48	87	11	2	13.2	1.8	0.3
4	28	65	31	4	5.8	2.8	0.4
5	12	78	20	2	3	0.8	0.1
Average					6.88	1.98	0.16
Deviation					3.83	0.99	0.16
E.P.M.					1.92	0.50	0.08

Table 24 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Subcutaneous injection of 50 μg of wild tobacco pollen grains (100 μL per animal, in a saline solution with 5 mg of aluminum hydroxide as adjuvant), followed by a *booster* in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure through intrapleural injection of 20 μg of pollen grains in a volume of 100 μL of saline solution per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	22	78	21	1	5.5	1.5	0.1
2	40	71.5	26.5	2	8.6	3.2	0.2
3	16	78	21	1	4.2	1.1	0.1
4	19	88	12	0	5.7	0.8	0
5	19	81	10	9	5.2	0.6	0.6
6	24	75	17	8	5.9	1.3	0.6
Average					12.96	1.43	0.26
Deviation					6.17	0.92	0.28
E.P.M.					2.76	0.41	0.12

Table 25 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Subcutaneous injection of 100 μg of wild tobacco pollen grains (100 μL per animal, in saline solution with 5 mg of aluminum hydroxide as adjuvant), followed by a *booster* in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure through intrapleural injection of 20 μg of pollen grains in a volume of 100 μL of saline solution per animal.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	32	81	16	3	8.81	1.74	0.33
2	15	80.5	19	0.5	4.11	0.97	0.03
3	19	84	15	1	5.11	0.91	0.06
4	27	69	27	4	5.96	2.33	0.35
5	23.25	78	22	0	6.89	1.9	0
6	23.25	80	9	11	6.32	0.7	0.87
Average					6.20	1.43	0.27
Deviation					1.61	0.66	0.33
E.P.M.					0.72	0.29	0.15

Example 9 – Immune response of rats subjected to subcutaneous injection of pollen grains, followed by a *booster* – challenge through subplantar injection of 20 μg of pollen grains

Two groups of animals were subjected to a subcutaneous injection (100 μL per animal) of pollen grain suspensions in the following conditions:

1 – Subcutaneous injection of 50 μg of pollen grains in a saline solution and alumminum hydroxide as adjuvant (5 mg per animal); and

2 – Subcutaneous injection of 100 μg of pollen grains in a saline solution and alumminum hydroxide as adjuvant (5 mg per animal).

One week after these subcutaneous injections (day 7) the groups of animals were subjected to a new subcutaneous injection (*booster*) in the same respective pollen grain concentrations, but without an adjuvant. After one week (day 14), all animal groups were challenged by sub plantar injection (50 μL) of 20 μg suspensions of pollen grains. There was no appearance of edemas on the sole of the paws/feet in any of the evaluated conditions.

Example 10 – Immune response of rats subjected to intranasal instillation of pollen grains, followed by a *booster* – challenge through subplantar injection of 20 μg of pollen grains

Four groups of animals were subjected to an intranasal instillation (50 μL per nostril) of pollen grain suspensions under the following conditions:

1 – Intranasal instillation of 50 μg of pollen grains in saline solution;

2 – Intranasal instillation of 25 μg of pollen grains in saline solution;

3 – Intranasal instillation of 50 μg of pollen grains in saline solution and aluminum hydroxide as adjuvant (5 mg per animal); and

04 – Intranasal instillation of 25 μg of pollen grains in saline solution and aluminum hydroxide as adjuvant (5 mg per animal).

One week after these nasal instillations (day 7) the animal groups were subjected to another intranasal administration (*booster*) with the same respective concentrations of pollen grains, but without adjuvants. After one week (day 14), all animal groups were challenged by a sub plantar injection (50 μL) of suspensions of 20 μg of pollen grains. There was no occurrence of paw/feet edemas in any of the evaluated conditions.

Example 11 – Immune response of rats subjected to consecutive intranasal instillations with WT and GM tobacco pollen grains

Groups of animals were subjected to intranasal instillations (50 µL per nostril) of wild pollen (WT) suspensions or transgenic pollen suspensions (GM – whose pollen grains contain the GUS protein) in the concentrations of 0, 5 and 50 total pollen µg per animal. Three instillations were performed with intervals of 7 days, after which a broncho-alveolar washing as well as a cellular counting was performed

Table 26 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Nasal instillation of a saline solution (control of 50 µL per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	7	70	0	30	0.123	0	0.053
2	6	96	0	4	0.144	0	0.006
3	10	60	0	40	0.15	0	0.1
Average					0.138	0	0.053
Deviation					0.014	0	0.047
E.P.M.					0.01	0	0.033

Table 27 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Nasal instillation of 5 µg of wild tobacco pollen grains – WT – (in a saline solution, instillation of 50 µL per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	54	51	0	49	0.689	0	0.662
2	50	48	0	52	0.6	0	0.65
3	27	55	0	45	0.371	0	0.304
4	28	43	0	57	0.301	0	0.399
Average					0.49	0	0.503
Deviation					0.184	0	0.18
E.P.M.					0.106	0	0.104

Table 28 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Nasal instillation of 5 μg of transgenic tobacco pollen grains – GM – (in a saline solution, instillation of 50 μL per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	48	30	0	70	0.36	0	0.84
2	29	58	0	42	0.421	0	0.305
3	75	37	1	62	0.694	0.019	1.163
4	39	63	0	37	0.614	0	0.361
Average					0.522	0.005	0.667
Deviation					0.157	0.009	0.408
E.P.M.					0.091	0.005	0.236

Table 29 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Nasal instillation of 50 μg of wild tobacco pollen grains – WT – (in a saline solution, instillation of 50 μL per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	172	4	0	96	0.172	0	4.128
2	24	51	0	49	0.306	0	0.294
3	109	20	0	80	0.545	0	2.180
4	39	66	0	34	0.644	0	0.332
5	104	56	0	44	1.456	0	1.144
Average					0.624	0	1.615
Deviation					0.216	0	1.6
E.P.M.					0.25	0	0.8

Table 30 – Experimental conditions of pollen grain exposure to *R. novergicus* Wistar rats and their respective results. Nasal instillation of 50 μg of transgenic tobacco pollen grains – GM – (in a saline solution, instillation of 50 μL per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.

N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	31	92	0	8	0.713	0	0.062
2	65	70	0	30	1.138	0	0.488
3	22	44	3	53	0.242	0.02	0.292
4	40	48	0	52	0.480	0	0.52
5	35	55	0	45	0.481	0	0.394
Average					0.610	0.003	0.351
Deviation					0.338	0.007	0.184
E.P.M.					0.169	0.004	0.092

The results of tables 26 – 30 are better visualized in Figure 3, panels D - F, and indicate that in none of the pollen grain concentrations administered by intranasal means any significant alteration in the cell counts (which would be typical of allergic-inflammatory reactions) occurred,. The statistically significant difference among the mononuclear cells and neutrophil counts after the administration of pollen grains WT and GM (Figure 3, panels D and E) is surprising and favorable in regard to the present invention's purposes, since it demonstrates that the pollen grains with modified composition (GM), when used in concentrations of 50 μg, would have less potential to induce inflammation in the broncho-alveolar cavity than wild tobacco pollen grains (WT).

Example 12 – Immune response of rats subjected to different programs of pollen grain administration – measurement of the total seric IgG through ELISA

Table 31 shows the seric IgG concentration data of the animals subjected to the conditions described in Example 6, tables 12 – 15.

Table 31 – Concentration data of the total seric IgG, in μg/mL, in serum samples of animals subjected to three consecutive intranasal administrations, with an interval of one week between each application of wild type tobacco pollen grain (WT) suspensions in different concentrations.

Animal	Saline solution	WT 5 μg	WT 50 μg	WT 500 μg
1	1125.6	1441.5	1123.3	1012.7
2	1367	1337.7	1459.5	1461.8
3	1376	1084.9	1414.4	1152.6
4	1545.3	836.7	1511.4	918
5	1504.6			1015
6				1488.9
N	5	4	4	6
Average	1383.7	1175.2	1377.2	1174.8
Deviation	164.1	270.8	173.8	244.7
E.P.M.	82.0	156.4	100.4	109.4

Table 32 shows the seric IgG concentration data of the animals subjected to the conditions described in Example 7, tables 16 and 19 - 21.

Table 32 – Concentration data of the total seric IgG, in μg/mL, in serum samples of animals subjected to an intrapleural injection with different concentrations of wild type tobacco pollen grains (WT), with or without adjuvant ($AlOH_3$), followed by a *booster* one week later, under the same conditions and challenge, and, one week thereafter by an intrapleural injection of 10 μg of pollen grains.

Animal	Saline solution	500 μg	50 μg + $AlOH_3$	500 μg + $AlOH_3$
1	942.8	956.3	981.1	951.8
2	807.4	665.2	906.7	1037.5
3	1024	836.7	678.8	1367
4	784.8	884.1	1100.7	960.8
5			954.1	1673.9
N	4	4	5	5
Average	889.8	835.6	924.3	1198.2
Deviation	113.5	123.8	154.8	315.3
E.P.M.	65.5	71.5	77.4	157.7

Table 33 shows the seric IgG concentration data of the animals subjected to the conditions described in Example 11, tables 26 - 30.

Table 33 – Concentration data of the total seric IgG, in μg/mL, in serum samples of animals subjected to 3 (three) consecutive intranasal

administrations, with an interval of one week between each application of wild type tobacco pollen grain (WT) suspensions or genetically modified (GM) in different concentrations.

Animal	Saline solution	WT 5 µg	WT 50 µg	GM 5 µg	GM 50 µg
1		1098.5	1069.1	967.6	523.1
2	1130.1	1457.3	1247.4	1062.4	784.8
3	1470.8	1078.2	931.5	1299.3	978.9
4	1283.5	1367	958.6	1048.8	893.1
5	1479.8	723.9	1103		744.2
N	4	5	5	4	5
Average	1341.1	1145.0	1061.9	1094.5	784.8
Deviation	167.2	287.6	126.3	142.8	172.9
E.P.M.	96.6	143.8	63.2	82.4	86.4

Example 13 – Analysis of the immune response of rats subjected to consecutive subcutaneous injections of pollen grains, recognized as allergens (*Betula fontinallis*)

Aiming to subject the animals to conditions in which a inflammatory and/or allergic response would be more likely, so as to eliminate the hypothesis that the animals utilized in the tests would not be responsive, new trials were conceived with the use of pollen grains reported in literature to be associated with allergic events (Ahlholm *et al*, "Genetic and Environmental Factors Affecting the Allergenicity of Birch (*Betula pubescens ssp. czerepanovii* [Orl.] Hamet-ahti) Pollen" *Clin. Exp. Allergy* **28**:1384-1388, 1998). In this sense, the pollen grains of *Betula fontinallis* were commercially acquired and utilized in experiments with rats as follows:

Groups of 5 (five) animals were subjected to the exposure of pollen grains in the following conditions:

Group 1 – Subcutaneous injection (100 µL per animal):

- *Betula* pollen suspension (100 µg/animal) in saline solution;

- *Betula* pollen suspension (100 µg/animal) in saline solution + $Al(OH)_3$ (5 mg/animal); and
- Wild tobacco pollen suspension – WT (100 µg/animal) in saline solution + $Al(OH)_3$ (5 mg/animal).

Group 2 – Instillation (50 µL per nostril):

- *Betula* pollen suspension (100 µg/animal) in saline solution + $Al(OH)_3$ (5 mg/animal).

After eight (8) weeks of consecutive exposures under the above-described conditions, Group 1 was subjected to a challenge, by injection into the *footpad*, of 50 µg of pollen grain suspension (50 µL) and measurement of the animal's feet volume in a plethysmograph. The results of Table 34 indicate the occurrence of edemas in the animals subjected to subcutaneous injections of WT tobacco pollen and, to a lesser degree, with *Betula* pollen.

Table 34 – The volumes, in ml, of the right (control) and the left paws/feet (challenge) were measured in a plethysmograph. The rats were subjected to 8 (eight) consecutive subcutaneous injections with pollen grains under the above-described conditions, followed by a challenge through a sub plantar injection of 50 µg of pollen grain suspension (50 µL per paw). The presented averages represent the volume differences between the left and right paws.

Tobacco WT + $Al(OH)_3$		***Betula* in saline solution**		***Betula* + $Al(OH)_3$**	
Right	**Left**	**Right**	**Left**	**Right**	**Left**
1.23	1.79	1.29	1.54	1.24	1.36
1.23	1.80	1.35	1.58	1.38	1.28
1.30	1.75	1.28	1.48	1.31	1.34
1.20	1.30			1.40	1.42
				1.32	1.29
Average	0.420		0.227		0.008
Deviation	0.220		0.025		0.081
E.P.M.					

After eight consecutive weeks of nasal instillations in the animals of Group 2, broncho-alveolar lavages were used to count the cells under the

above-described conditions. The cell counts indicated that there was no change in relation to the control animals. Taken together these results point to the following conclusions: (i) the allergic response to subcutaneously injected pollen grains only occurs after an elevated number of consecutive exposures and tends to be higher in the absence of an adjuvant; (ii) it was not possible to detect typical alterations of allergic responses in any of the nasal instillation conditions in rats. Since edemas indeed occurred under the subcutaneous injection condition (with WT tobacco pollen and also with *Betula* pollen) and the absence of typical allergic alterations through instillation in all of the conditions evaluated in the present invention, the results indicate that there seems to be a relation between the antigen presentation route and the pattern of response.

Example 14 – Immune response of rats subjected to different programs of pollen grains administration – detection of specific seric IgG against of pollen grain proteins through Western Blot

The formation of specific seric IgG against pollen grain proteins was monitored through Western Blot, in which total extracts of pollen WT (wild) and GM (containing the GUS enzyme) proteins were separated through electrophoresis, followed by the transference of the bands to a nitrocellulose membrane. The serum of the animals subjected to three consecutive instillations, in intervals of one week each, with pollen WT or GM, in *pool*, were then incubated separately, with said membrane (previously cut into pieces to allow the separation of the samples), which, after being washed, was incubated in the presence of secondary anti-rat IgG antibodies marked with alkaline phosphatase. The colorimetrical reaction with adequate substrates indicated the detection of specific IgG's against pollen grain proteins. As shown on Table 35, the serum of the animals instilled with tobacco pollen grains "WT" and also in the serum of the animals instilled with tobacco pollen grains "GM" revealed the presence of IgG type antibodies specific against proteins of said pollen grains. Taken together, the results of these experiments point to the feasibility of the

use of pollen grains as modulators of the immune response in mammals. More specifically, the results point to the feasibility of the use of whole pollen grains as stimulators of protective response (formation of specific seric IgG against pollen grain proteins) in mammals.

Table 35 – Detection, through Western Blot, of specific seric IgG against proteins from tobacco pollen grains WT and GM.

Condition of exposure →	**Saline solution**	**Pollen WT 5 µg**	**Pollen GM 5 µg**	**Pollen GM 50 µg**
seric IgG	-	+	+	-

The approach using pollen grains has, besides other advantages mentioned in the present invention, the important characteristic of requiring very small amounts of vegetal material for immunization. The results of Table 35 show the formation of specific seric IgG against proteins present in pollen grains after three (3) administrations of 5 µg of pollen grains. Even though no study was so far performed in order to define the minimum amounts necessary to induce the immune response observed on Table 35, the present results offer a reasonable base to estimate the amount of pollen grains that would be necessary for the immunization in other species, such as humans. It is known that the human immune system is considered to be from 100 to 1.000 times more sensitive than that of murines, which means that, by maintaining the proportion between the administered amount and the body weight, just some dozens or hundreds of square meters of cultivation would be sufficient to produce vaccines for entire populations. This allows, among other things, the adoption of simple containment measures in order to decrease biosafety concerns. Further, it is convenient to remember that several other strategies for pollen grain containment are available, such as irradiation, the use of suicidal genes, and infertility barriers. The present invention's strategy is particularly applicable for animal vaccinations, being also useful to fight epidemics and

prevent or remedy situations which might result from the use of biological weapons or terrorist attacks.

Example 15 – Obtaining genetic constructions for the transformation of plants destined to produce pollen grains with heterologous polypeptides on their external surface

In another preferred embodiment of the present invention, the presence of heterologous polypeptides on the external surface of pollen grains is desirable because it is related to the use of pollen grains in *in vitro* antigen-antibody type reactions, that is, reactions used for *in vitro* immune diagnosis. In order to evaluate this applicability, *A. thaliana* plants were transformed with gene constructions which would direct the expression of heterologous polypeptide in a subcellular localization compatible with its presence on the external surface of the pollen grains.

In order to fulfill these requirements, in one of the preferred embodiments of the present invention, the coding gene of the heterologous polypeptide in question is translationally fused to the coding sequence of the AtGRP17 gene (SEQ 1), while said fusion is controlled by at least part of the promoter region (SEQ 2) of the AtGRP17, this promoter being able to direct the gene fusion expression in the anther's tapetum. In order to prepare said gene construction, the following steps were taken:

For the amplification of the promoter region of the AtGRP17 and its ORF specific oligonucleotides were used: RR1f (5'ATA AAG CTT TTT CTC TGT TTT TGT CCG TGG AAC) and RR2r (5'ATA CCA TGG CAC GTG ATT CGG TGG AAG TCC TGC C). The plasmid pCO27 (described by Oliveira *et al* "Inflorescence-specific genes from *Arabidopsis thaliana* encoding glycine-rich proteins". *Plant J.* **3**:495-507, 1993; Franco *et al.*, "Distal regulatory regions restrict the expression of *cis*-linked gene to the tapetal cells". *FEBS Letters* 25965:1-6, , 2002) was used as target for the amplification, by PCR, of the promoter region and of the AtGRP17 ORF. By using the oligonucleotides RR1f and RR2r the product of amplification ProAtGRP67 (Figure 6) was obtained

and, after cleavage with the enzymes XbaI and NcoI, was linked to plasmid pCambia cleaved with the same enzymes (Figure 5), thus originating the construction pProAtGRP17_GUSGFP (Figure 7).

Example 16 - Transformation of *A. thaliana* and analysis of the heterologous polypeptide's localization

The gene construction of the previous example was introduced in *E. coli* and in *Agrobacterium tumefasciens* so as to later transform *A. thaliana*. Plant transformation confirmation was performed through the extraction of genomic DNA by methodologies known to skilled in the art and by PCR-mediated amplification of the heterologous regions introduced into the transformed plant. Plant transformation was also confirmed by the expression analysis of GUS enzyme activity in the intented subcellular localiztions, by means of a colorimetric reaction using X-Gluc as substrate and the corresponding blue color formation. Plant tissue samples were observed by stereoscopic and optical microscopy.

The gene construction (Figure 7) containing the reporter genes *gus* and *gfp*, was introduced in *E. coli* by eletroporation and in *A. tumefasciens* by thermal shock. The cloning was confirmed by the analysis of the expected profile of fragments generated by the digestion with restriction enzymes (Figure 8). *A. thaliana* inflorescences were inoculated with cultures of *A. tumefasciens* containing said gene construction (transformation technique called "floral dip", described by Clough & Bent "Floral dip: a Simplified Method for *Agrobacterium*-mediated Transformation of *Arabidopsis thaliana*" *The Plant Journal* **16**(6):735-743, 1998). The seeds generated after that procedure were collected and sown on plaques containing a selective medium comprising hygromicyn. The plantlets selected from the plaques were transplanted into the soil in phytotron, where they were cultivated in conditions which favor self-fertilization. Analysis of these plants' inflorescences by microscopy (Figure 9 A – D) confirmed the presence of GUS activity in the expected subcellular localization (by means of the

corresponding colorimetrical reaction described in Example 2). More specifically, the activity of the GUS enzyme was only detected in the later stages of the anther's development (Figure 9 A), and still more specifically on the anther's tapetum (Figure 9 C).

Example 17 – Detection of the heterologous polypeptide on the surface of pollen grains

The transformation of *A. thaliana* plants according to Example 16 resulted in the generation of 11 lineages effectively producing heterologous polypeptides in the pollen grains. Those lineages were cultivated in phytotron and self-polinated so as to allow the later obtention of hundreds of seeds. Seeds of each one of the lineages were sown directly into the soil and the resulting plants were cultivated in phytotron. After inflorescence formation entire flowers were collected in Eppendorf tubes with the help of a scalpel. 100 µL of phosphate buffer 0.1 M pH 7 was added to said tubes, which were then vortexed in order to allow the formation of homogeneum pollen grain suspensions. Afterwards, 1 µL of the X Gluc reagent was added and, 30 minutes at 37°C thereafter, the presence of the GUS enzyme triggered the formation a blue-colored complex. Fractions of these suspensions were then collected with micropipette and put on glass slides for observation under the microscope. The result of that observation (Figure 9 D) indicated the presence of the active heterologous polypeptide (GUS enzyme) in the pollen grain. These results point collectively to the feasibility of the use of whole pollen grains as reagents for immune diagnosis.

The skilled persons will understand from the present description that any heterologous polypeptide can be produced by the processes of the present invention, including post-translationally modified polypeptides, such as glycosilated proteins and the like. Similarly, combinations of heterologous polypeptides or translational fusions of heterologous polypeptide segments may

be produced by the teachings of the present invention, including, but not limiting to, at least part of polypeptides derived from eukaryotic organisms such as mammals including humans, plants, parasites, fungi or derived from procariotic organisms such as bacteria or even viruses, as well as combinations thereof, regardless od being natural or synthetic polypeptides. Therapeutic peptides preferred for the purposes of the present invention include peptidic hormones, cytokines, interleucins, antibodies (and/or fragments thereof) and combinations thereof. The heterologous polypeptides produced by these processes may be used in several immunoreactions, including the immunomodulation of mammals and *in vitro* immunodiagnostic reactions. The referred immunomodulation may have an immunotherapic, immunoprotective or vaccination purpose, according to the chosen immunogen and/or according the combination of the chosen immunogens.